



Review

The Role of Nrf2 in the Antioxidant Cellular Response to Medical Ozone Exposure

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Abstract: Ozone (O₃) is a natural, highly unstable atmospheric gas that rapidly decomposes to oxygen. Although not being a radical molecule, O₃ is a very strong oxidant and therefore it is potentially toxic for living organisms. However, scientific evidence proved that the effects of O₃ exposure are dose-dependent: high dosages stimulate severe oxidative stress resulting in inflammatory response and tissue injury, whereas low O₃ concentrations induce a moderate oxidative eustress activating antioxidant pathways. These properties make O₃ a powerful medical tool, which can be used as either a disinfectant or an adjuvant agent in the therapy of numerous diseases. In this paper, the cellular mechanisms involved in the antioxidant response to O₃ exposure will be reviewed with special reference to the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) and its role in the efficacy of ozone therapy.

Keywords: ozone therapy; oxidative stress; proteostasis; mitochondria; inflammation; adipose biology; cancer

1. Ozone in the Biomedical Field

Ozone (O₃) is a natural gas forming from dioxygen (O₂) by the action of ultraviolet light and electrical discharges in the atmosphere; however, O₃ is highly instable, rapidly breaking down to its diatomic allotrope. For this reason, it occurs in very low amounts in the atmosphere compared to O₂. O₃ is known as a strong oxidant being at the same time a dangerous respiratory hazard and pollutant contributing to several diseases (recent review in [1–5]); it is also a powerful oxidizing agent with manifold industrial applications (e.g., as disinfectant, deodorizer, cleaning and bleaching agent) [6–9] and consumer implementations (e.g., as food additive, or air and water purifier) [10–13].

During the last decades, O₃ has increasingly been applied as O₂-O₃ mixtures also in complementary/adjuvant medicine. Actually, the medical use of O₃ dates back to the 18th century [14], when the Dutch physicist Martin van Marum discovered that an electric spark through the air gave rise to a gas with a typical odour and strong oxidizing properties. However, the link between dioxygen chemical modification and formation of the oxidant gas was only understood in 1840 by the German chemist Christian Friedrich Schönbein who called this gas “ozone” (from the Greek word ὄζειν, “to smell”) and revealed its capability to interact with organic compounds. Subsequently, various generators of O₃-O₂ mixtures were built, and the first studies on the biological effects of O₃ were performed. Consequently, in the 19th century, O₃ started to be used for therapeutic purposes, especially in treatment of allergy, and even Nikola Tesla patented an O₃ generating system for medical use. During the last century, O₃ was administered by different ways (gaseous O₃ inhalation, injection or bags; auto-hemotherapy; ozonated water or oil) to treat a number of diseases (e.g., anaemia, diabetes, fever, gangrene, syphilis, tetanus, tuberculosis). Starting from late 1970s, O₂-O₃ therapy had a great

development all over the world, thanks to the refinement of administration techniques and protocols, and to the collaborative work of some European medical societies. This contributed significantly to the set-up of regulatory requirements for O₃ application, including the production of medical O₃, the use of appropriate disposable materials, and the definition of concentration, dosages, and treatment frequency in relation to the disease [15]. In addition, based on clinical practice and according to the principle of “hormesis” (i.e., “the beneficial effect of a low level exposure to an agent that is harmful at high levels”) [16], a progressive lowering of O₃ dosage has been adopted, relaying its beneficial effects to the cascade of metabolic events triggered by the initial, mild oxidative stress [17].

In parallel to clinical advancement, an increasing number of scientific reports have been published on the biomedical effects of O₃ as adjuvant/alternative therapeutic approach for e.g., pain management [18–22], gastrointestinal diseases [23–27], lung diseases [28–31], diabetes [32–37], ischemia [38–43], cancer [44–46], infective diseases [47–50], dentistry [51–56].

However, despite the wide scientific demonstration of the multiple therapeutic applications of O₂-O₃ treatment, the biological mechanism(s) responsible for the positive effects of O₃ administration have been only partially elucidated.

2. Ozone-Induced Activation of the Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2)

Exposure to toxic levels of atmospheric O₃ induces injury and inflammation through activation of the redox sensitive nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is one of the main players in transcribing pro-inflammatory cytokines and, in turn, in increasing the expression of several proteins involved in the antioxidant response [47,57–65]. Among these proteins, Nrf2 has been demonstrated to play a crucial role in the activation of cytoprotective antioxidant genes against atmospheric pollutant-induced toxicity [66].

Similarly, exposure to low O₃ concentrations for therapeutic purposes was found to act on Nrf2. Ozonated serum was able to activate Nrf2 in a dose dependent manner and to subsequently induce the expression of heme oxygenase 1 (HMOX1) and nicotinamide adenine dinucleotide phosphate (NAD(P)H) quinone oxidoreductase 1 (NQO1) in endothelial cells [67]. Systemic O₃ treatment in healthy volunteers increased the levels of Nrf2 in peripheral blood mononuclear cells with consequent enhanced activity of superoxide dismutase and catalase [68]. The application of ozonated saline in an in vitro human keratinocyte model of wound healing proved the activation of Nrf2 pathways resulting in the increased expression of the HMOX1 gene [69]. Systemic O₃ administration in rats with adenine-induced chronic kidney disease inhibited the NF-κB pathway and induced Nrf2 activation: this resulted in the up-regulation of antioxidant enzymes and the down-regulation of inflammatory cytokines in the kidney, with reduction of the renal insufficiency and tubulointerstitial injury [70]. Rectal insufflation of O₃ to patients affected by multiple sclerosis increased Nrf2 phosphorylation and casein kinase 2 (CK2) expression in mononuclear cells, thus improving the activity of antioxidant enzymes and reducing the levels of pro-inflammatory cytokines [71]. The systemic administration of O₂-O₃ was beneficial in the rat model of streptozotocin-induced pancreatic damage by increasing the endogenous Nrf2 and glutathione-S-transferase (GST) in the pancreatic tissue [72]. O₃ treatment in rats induced oxidative preconditioning by activating Nrf2, thus protecting the lung and myocardium from the ischemia-reperfusion injury, a major cause of cardiac and respiratory dysfunction during cardiovascular surgery, heart transplantation and cardiopulmonary bypass procedures [73,74]. O₂-O₃ therapy proved to be beneficial also for the treatment of diabetic complications and spinal pain by activating various antioxidant pathways involving hypoxia inducible factor-1α (HIF-1α), nuclear factor of activated T-cells (NFAT), activated protein-1 (AP-1) pathways, and Nrf2 [75].

Based on these observations, it was hypothesized that the antioxidant and anti-inflammatory effects of low O₃ concentrations involve activation of Nrf2, which is thus considered as a key factor for the efficacy of O₂-O₃ treatments [76–78].

3. Biological Role of Nrf2 and Its Activation by Ozone Treatment

Nrf2 was first cloned in 1994 and identified as a member of the human cap'n'collar basic-region (CNC) leucine zipper transcription factor family [79], which also includes nuclear factor erythroid 2 (NF-E2), nuclear factor erythroid 2-related factor 1 (Nrf1), nuclear factor erythroid 2-related factor 3 (Nrf3), BTB domain and CNC homolog 1 (BACH1), and BTB domain and CNC homolog 2 (BACH2). Nrf2 dimerizes with the small musculoaponeurotic fibrosarcoma (Maf) proteins to bind and mediate the transcription at a consensus sequence containing an AP-1 core motif [80]; this latter was identified on the promoter of the rat GST *Y α* subunit gene [81] and thereafter referred to as Antioxidant Responsive Element (ARE) [82]. Under basal conditions, Nrf2 is expressed at very low level, and is mainly sequestered in the cytoplasm by its specific inhibitor, Kelch-like ECH associated protein-1 (Keap-1) that also promotes its rapid degradation [83]. The effectiveness of this mechanism allows a rapid turnover of Nrf2, which displays a half-life of a few minutes. Under specific stimuli, Nrf2 dissociates from Keap1, translocates into the nucleus and transactivates ARE-driven genes [84]. By combining sub-nuclear tracking of Nrf2 localization and functional genetic engineering we have recently demonstrated that mild ozonisation increases the nuclear translocation of Nrf2 at transcriptionally active chromatin sites, inducing the Nrf2-mediated Keap1-dependent transcription of ARE-driven genes [85].

Twenty-five years of studies have contributed to identify more than two hundred ARE genes, whose expression is regulated by the Nrf2 transcriptional activity. These genes encode for proteins involved in a multitude of vital biological functions which include protein homeostasis, oxidative stress response, detoxication, DNA repair, proliferation, autophagy, mitochondrial biogenesis and function, inflammation, and the metabolism of lipids, carbohydrates and amino acids [86,87]. The impact of Nrf2 on transcriptome is amplified by the fact that Nrf2 may directly regulate or bidirectionally cross-talk with many other transcription factors such as Notch1 [88], the aryl hydrocarbon receptor (AhR) [89], the CCAAT/enhancer-binding protein (C/EBPB) [90], the peroxisome proliferator-activated receptor gamma (PPAR γ) [91], the retinoid X receptor alpha (RXRA) and NF- κ B. Finally, multiple lines of evidence have shown that Nrf2 activation is part of the retrograde response aimed at restoring mitochondrial function after stress insults, and that the impairment of Nrf2 functions is a hallmark of many mitochondrial-related disorders [92–95].

Some of the cell functional pathways that are dependent on Nrf2 activation (Figure 1) are summarized in the following paragraphs.

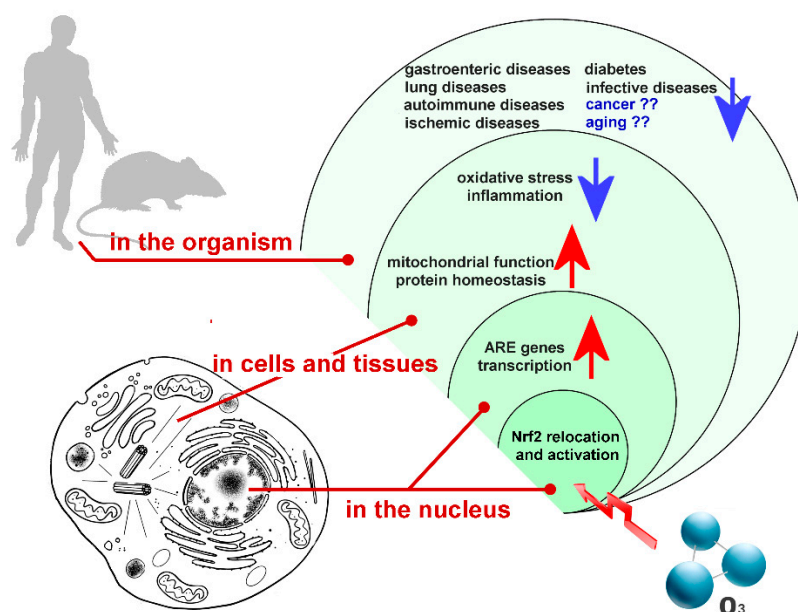


Figure 1. Main functional pathways depending on Nrf2 activation induced by low ozone concentrations. Arrows indicate up- (red) or down- (blue) regulation.

3.1. Nrf2 and Oxidative Stress

Oxidative stress is due to the accumulation of reactive oxygen species (ROS) (O_2^- , H_2O_2 and $\bullet OH$), that are generated as by-products of either physiological or exogenous stress factors (such as, e.g., the ionizing radiations). Although ROS have been recognized in the last years as functionally significant signalling molecules for the modulation of the immune system, they have long been seen as harmful factors with detrimental effect on cell homeostasis. For example, ROS are strong inducers of DNA damage [96] that irreversibly compromises cell functions and might stimulate neoplastic transformation [97]. Nrf2 prevents oxidative stress through the transcription of antioxidant enzymes, such as the catalytic and modulatory subunits of glutamate cysteine ligase (GCL), glutathione peroxidases (GPX2 and GPX4), glutathione reductase (GSR), peroxiredoxins (PRDX1 and PRDX6), thioredoxin 1 and thioredoxin reductase 1 (TXN1 and TXNRD1), HMOX1, and biliverdin reductase (BVR) [87,98–100]. Accordingly, we demonstrated that mild ozonisation induces modulation of genes involved in the cell response to stress (HMOX1; excision repair cross-complementation group 4, ERCC4; cyclin-dependent kinase inhibitor 1A, CDKN1A) and in the transcription machinery (CTD small phosphatase 1, CTDSP1) [101].

3.2. Nrf2 and Proteostasis

Oxidative stress is one of the major drivers of protein misfolding as it induces protein oxidation. Misfolded or unfolded proteins accumulate as insoluble inclusions and aggregates in the cytoplasm and the cell nucleus, and are the hallmark of multiple aging-related neurodegenerative and metabolic disorders [102]. Nrf2 promotes the clearance of oxidized or otherwise damaged proteins through the two major pathways of protein degradation, i.e., the ubiquitin proteasome system and autophagy. Nrf2 directly targets the transcription of many proteasomal genes [103,104] and of the proteasome maturation protein (POMP) [105]. Nrf2 also supports autophagy either directly (by regulating the transcription of key autophagic genes, such as autophagy related ATG 5 and 7, unc-51-like autophagy activating kinase (ULK) 1 and 2, and the autophagy transporter ubiquitin-binding protein p62, sequestosome-1 (SQSTM1) [106], or indirectly through the activation of mammalian target of rapamycin (mTOR), a master regulator of protein synthesis and autophagy [107]. Consistently, O_3 treatment proved to promote wound healing by increasing the migration of fibroblasts via the phosphoinositide 3-kinase (PI3K)/protein-kinase B (Akt)/mTOR signalling pathway [48], and to increase the level of autophagy in a chondrocyte model of osteoarthritis through the activation of AMP-activated protein kinase (AMPK)/mTOR [108].

3.3. Nrf2 and the Mitochondrial Function

The classic view of mitochondria as semi-independent organelles has recently been integrated by the evidence that, in response to environmental changes, they may coordinate inter-organelle signalling pathways (mitochondrial retrograde response) that ultimately instruct nuclear gene expression [109–111]. Maintenance of an efficient mitochondrial function is crucial to preserve cell homeostasis, as proven by the evidence that mitochondrial stimulation by mild-stress treatments (mitohormesis) [112] underlies the beneficial effects of life-extending interventions such as dietary restriction [113,114]. In contrast, mitochondrial dysfunction is ontologically linked (as either cause, con-cause or consequence) to aging and aging-related diseases [115,116]. Nrf2 has been shown to stimulate mitochondrial biogenesis through the activation of nuclear respiratory factor-1 (NRF-1) in cardiomyocytes [117,118]. Cellular respiration and ATP synthesis are impaired in conditions of Nrf2 deficiency and increased upon Keap1 loss of function [119,120]. Nrf2 is functionally linked through a positive feedback loop to Sqstm1/p62, which localizes to the mitochondria and enhances the mitochondrial transcription factor A (TFAM), a master regulator of mitochondrial biogenesis [121,122]. In addition, Nrf2 has been identified as a crucial mediator of the mitochondrial biogenesis induced by acetyl-carnitine (ALCAR), nitric oxide (NO) and resveratrol [123,124]. Accordingly, mild ozonisation

was found to affect mitochondria by increasing the length of the mitochondrial cristae and the content of mitochondrial heat-shock protein 70 [125], while O₃ treatment was proven to reduce mitochondrial damage in a rat heart following ischemia-reperfusion [73] as well as in a rat brain and cochlea following noise-induced hearing loss [126].

3.4. Nrf2 and Inflammation

Inflammatory pathways protect from exogenous harmful stimuli and, to some extent, from intrinsic dysregulation of cell proliferation that may lead to neoplastic growth. However, inflammation may also take place during aberrant self-reactivity in autoimmune diseases [127], while a low but chronic level of inflammation in the absence of real exogenous or endogenous threats is a common hallmark of aging (inflammaging) and aging-related diseases [128,129]. Nrf2 is able to modulate inflammation through multiple mechanisms, such as the regulation of redox homeostasis and the suppression of pro-inflammatory genes, either directly or through the interaction with NF-κB. Inflammation increases local and systemic ROS level while ROS enhance inflammation [130]. The Nrf2-mediated ROS-homeostatic control is able to break this vicious cycle. Nrf2 reduces inflammation by preventing the recruitment of RNA polymerase II to start gene transcription of pro-inflammatory cytokines IL-6 and IL1β [131]. In addition, Nrf2 tunes gene expression in inflammatory macrophages through a bidirectional crosstalk with NF-κB transcription factor, and regulates the transcription of Nrf2 itself [132] that, in turn, inhibits the transcriptional activity of NF-κB [133]. NF-κB has long been recognized as a point of convergence of inflammation and aging, and the array of NF-κB-regulated genes largely overlaps with the targets of transcriptional/epigenetic regulators such as sirtuins that have been demonstrated to promote lifespan extension [134–136]. This notion once again corroborates the ontological link between inflammation and aging and allows speculation that O₃-induced activation of Nrf2 might be a potential tool to prevent aging and prolong lifespan.

3.5. Nrf2 and Adipose Biology

Oxidative stress is a crucial factor in adipocyte differentiation, and ROS are known to promote adipogenesis via insulin-mediated signal transduction [137,138]. As an antioxidant regulator factor, Nrf2 is involved in the adipogenic differentiation of mesenchymal stem cells [90,139,140], together with HMOX-1 [141]. A recent study on 3T3-L1 cells suggested that Nrf2 affects adipocyte differentiation by modulating the expression of the fibroblast growth factor 21 (FGF21) through the PPARγ, a master transcription factor regulating inflammation as well as adipogenesis and insulin sensitization [142]. Consistently, treating the adipose tissue with low O₃ concentrations proved to exert an adipogenic effect on human adipose-derived adult stem cells [143] in the absence of damage in differentiated adipocytes [144]; this allows foreseeing mild ozonisation as an adjuvant tool for tissue regeneration and engineering.

3.6. Nrf2 and Cancer

Unlike the vast majority of diseases that have an often unique pathogenic cause, cancer can be defined as multitude of possible pathologic states sharing the capability to subvert and redirect the regeneration and differentiation potential of cells and tissues toward abnormal limitless proliferation and growth. The Nrf2-mediated regulation of the biological processes described above has proven to exert beneficial effects in preventing, ameliorating or curing a multitude of diseases, but might turn detrimental in a cancer-related context. Nrf2 preserves cells from DNA damage, and therefore might help preventing the primary trigger of neoplastic transformation. However, hyperactivation of Nrf2 has been shown to support tumour progression by multiple ways [145,146]: for example, it may help incipient tumour cells to overcome oxidative stress that represents a barrier against neoplastic transformation and cancer initiation [147]. Also, Nrf2 hyperactivation supports aberrant cell proliferation by both inducing the metabolic switch towards anabolic pathways [148] and modulating mRNA translation [149]. Moreover, Nrf2 may promote tumour angiogenesis [150]. Finally, the

potent cytoprotective effect of Nrf2 activation may confer drug resistance to cancer cells [151]. Thus, the possible tumour-promoting effect of Nrf2 hyperactivation still remains a crucial issue, since oncological patients are frequently administered O₃ therapy due to its efficacy in reducing some adverse side-effects of the anti-cancer treatments [46,152,153].

4. Conclusions

As a whole, the direct and indirect molecular targets of Nrf2 delineates a complex network of biological processes that preserve cell homeostasis and promote cell reparative programs following chemical, physical, or biological stress [154]. The complexity of the Nrf2 functional network does not allow drawing an exhaustive molecular model that might explain the beneficial effects of O₃ in preventing or ameliorating diseases. Nrf2 activation exerts positive effects especially on diseases that have oxidative stress and inflammation as primary etiopathological events [155,156]. Therefore, it may be hypothesized that the therapeutic potential of Nrf2 activation as a consequence of mild ozonisation relies on the capability of Nrf2 to maintain redox homeostasis: this would prevent DNA damage, preserve proteostasis, and improve mitochondrial function while suppressing acute and chronic inflammation.

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Abbreviations

Nrf2	nuclear factor erythroid 2-related factor 2
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
HMOX1	heme oxygenase 1
CK2	casein kinase 2
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NQO1	nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1
GST	glutathione-S-transferase
HIF-1α	hypoxia inducible factor-1α
NFAT	nuclear factor of activated T-cells
AP-1	activated protein-1
CNC	cap'n collar basic-region
NF-E2	nuclear factor erythroid 2
Nrf1	nuclear factor erythroid 2-related factor 1
Nrf3	nuclear factor erythroid 2-related factor 3
BACH1	BTB domain and CNC homolog 1
BACH2	BTB domain and CNC homolog 2
Maf	musculoaponeurotic fibrosarcoma
ARE	Antioxidant Responsive Element
Keap-1	Kelch-like ECH associated protein-1
AhR	aryl hydrocarbon receptor
C/EBPB	CCAAT/enhancer-binding protein
PPARγ	peroxisome proliferator-activated receptor gamma
RXRA	retinoid X receptor alpha
ROS	reactive oxygen species
GCL	glutamate cysteine ligase
GPX	glutathione peroxidase
GSR	glutathione reductase
PRDX	peroxiredoxin

TXN1	thioredoxin 1
TXNRD1	thioredoxin reductase 1
BVR	biliverdin reductase
ERCC4	excision repair cross-complementation group 4
CDKN1A	cyclin-dependent kinase inhibitor 1A
CTDSP1	CTD small phosphatase 1
POMP	proteasome maturation protein
ATG	autophagy related
ULK	unc-51-like autophagy activating kinase
SQSTM1	sequestosome-1
mTOR	mammalian target of rapamycin
PI3K	phosphoinositide 3-kinase
Akt	protein-kinase B
AMPK	adenosine monophosphate-activated protein kinase
NRF-1	nuclear respiratory factor-1
TFAM	mitochondrial transcription factor A
ALCAR	acetyl-carnitine
NO	nitric oxide
FGF21	fibroblast growth factor 21

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